NASA-CR-196948

Final Technical Report for NASA Grant NAG2-782: "Skeletal Muscle Glucose Transport during Simulated Weightlessness"

Term of grant: 1992-1993 (extended to 1994)

Note: This document officially acts as a substitution for the semi-annual Status Reports)

Date of submission: September 20, 1994

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Final Technical Report, NAG2-782 Henriksen, Erik J.

This document will provide a brief summary of the major accomplishments achieved as a result of NASA support through grant NAG2-782. In the addendum to this document are reprints of all manuscripts which were published as a direct result of this support.

Summary of findings:

A. **Series 1:** Effect of insulin mimickers on glucose transport activity in unweighted skeletal muscle.

1. Published in: Henriksen, E. J., and L. S. Ritter. Effect of insulin-like factors on glucose transport activity in unweighted rat skeletal muscle. *J. Appl. Physiol.* 75: 820-824, 1993.

2. We have previously demonstrated that mechanical unweighting of the soleus muscle by hindlimb suspension leads to increases in insulin receptor binding, muscle/fat-specific glucose transporter (GLUT-4) protein levels, and insulin-stimulated glucose transport activity. This investigation used a novel approach to further evaluate the potential role of post-receptor binding mechanisms in this enhanced insulin effect following unweighting. Insulin-like growth factor I (IGF-I), vanadate, and phospholipase C (PLC) were used to stimulate glucose transport activity independently of insulin receptor binding. Soleus glucose transport activity (assessed by 2-deoxyglucose (2DG) uptake) was evaluated in vitro using soleus strips (~18 mg). Progressively increased responses to maximally effective doses of

insulin or IGF-I were observed after 3 and 6 days of unweighting compared to weight-matched controls. Enhanced maximal responses to vanadate (6 days only) and PLC (3 and 6 days) for 2DG uptake were also observed. The results of this study:

- provide evidence that post-insulin receptor binding mechanisms also play a role in the enhanced response of the insulin-dependent pathway for stimulation of glucose transport in unweighted skeletal muscle, and

- indicate that IGF-I action on glucose transport is included in this enhanced response in unweighted muscle.

B. **Series 2:** Effect of muscle contractions on glucose transport activity in unweighted skeletal muscle.

1. Published in: Henriksen, E. J., and L. S. Ritter. Effect of soleus unweighting on stimulation of insulin independent glucose transport activity. *J. Appl. Physiol.*74: 1653-1657, 1993.

2. As indicated above, unweighting of the rat soleus by tail-cast suspension results in increased insulin action on stimulation of glucose transport, which can be explained, at least in part, by increased insulin binding and enhanced GLUT-4 protein levels. Glucose transport is also activated by an insulin-independent mechanism stimulated by in vitro muscle contractions or hypoxia. Therefore, the purpose of this series of experiments was to determine if soleus unweighting leads

to an enhanced response of the insulin-independent pathway for stimulation of glucose transport. The hindlimbs of juvenile male Wistar rats were suspended by a tail-cast system for 3 or 6 days. As before, glucose transport activity was assessed in isolated soleus strips (~18 mg) using 2DG uptake. Insulin (2 mU/ml) had a progressively enhanced effect on 2DG uptake after 3 and 6 days of unweighting (+44% and +72% vs. control, both $P<0.001$). At these same time points, there was no difference between groups for activation of 2DG uptake by maximally effective treatments with contractions (10 tetani), hypoxia (60 min), or caffeine (5 mM). These results indicate that:

- the enhanced capacity for stimulation of glucose transport following soleus unweighting is restricted to the insulin pathway, with no apparent enhancement of the insulin-independent pathway.

Effect of insulin-like factors on glucose transport activity in unweighted rat skeletal muscle

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HENRIKSEN, ERIK J., AND LESLIE S. RITTER. *Effect of insulin-like factors on glucose transport activity in unweighted rat skeletal muscle.* J. Appl. Physiol. 75(2): 820–824, 1993.—We have previously demonstrated that mechanical unweighting of the soleus muscle by hindlimb suspension leads to increases in insulin receptor binding, muscle/fat-specific glucose transporter (GLUT-4) protein levels, and insulin-stimulated glucose transport activity. The present study used a novel approach to further evaluate the potential role of postreceptor binding mechanisms in this enhanced insulin effect after unweighting. Insulin-like growth factor I (IGF-I), vanadate, and phospholipase C were used to stimulate glucose transport activity independently of insulin receptor binding. Soleus glucose transport activity (assessed by 2-deoxyglucose uptake) was evaluated in vitro with soleus strips (~18 mg). Progressively increased responses to maximally effective doses of insulin or IGF-I were observed after 3 and 6 days of unweighting compared with weight-matched control strips. Enhanced maximal responses to vanadate (6 days only) and phospholipase C (3 and 6 days) for 2-deoxyglucose uptake were also observed. The results of this study *1*) provide evidence that post-insulin receptor binding mechanisms also play a role in the enhanced response of the insulin-dependent pathway for stimulation of glucose transport in unweighted skeletal muscle and *2*) indicate that IGF-I action on glucose transport is included in this enhanced response in unweighted muscle.

soleus; simulated weightlessness; 2-deoxyglucose uptake; insulin; insulin-like growth factor I; vanadate; phospholipase C

THE REMOVAL OF the weight-bearing function of rat skeletal muscle (known as unweighting) by means of hindlimb suspension has been successfully used as an earth-based model of weightlessness (for review see Ref. 26). One of the most striking metabolic adaptations of unweighted rat skeletal muscle is the development of an increased response to insulin for stimulation of glucose transport, demonstrated using in vitro muscle incubations (1, 11, 15, 16) and the perfused hindquarter (24). Concomitant with the development of this enhanced insulin action are increases in insulin receptor binding (1, 16) and in the expression of the muscle/fat-specific glucose transporter isoform (GLUT-4) (13). Both of these adaptive responses are likely to contribute to the enhanced response to insulin for activation of glucose transport after unweighting.

In addition to insulin itself, there are a number of agents that activate glucose transport in skeletal muscle through an insulin-dependent mechanism but that bypass the initial insulin receptor-binding step. These compounds include the hormone insulin-like growth factor I (IGF-I) (10, 23), which utilizes its own receptor system (6), and the insulin mimetic agents vanadate (Na_3VO_4) (3, 8, 14) and phospholipase C (PLC) (12, 22). Insulin mimickers have been used successfully to elucidate possible postreceptor mechanisms for altered insulin responses in skeletal muscle, such as the increased insulin sensitivity that develops after an acute bout of exercise (2) or the decreased action of insulin on glucose transport activity in denervated muscle (13, 23).

At present, the potential role of postreceptor mechanisms for the enhanced activation of the insulin-dependent pathway of glucose transport activity in unweighted muscle has not been addressed. Therefore, in the present study we examined the effect of 3 or 6 days of unweighting on glucose transport activity, as assessed by 2-deoxyglucose (2-DG) uptake, in soleus strips stimulated by maximally effective concentrations of insulin, IGF-I, vanadate, or PLC. Our data provide support for the existence of postreceptor binding mechanisms for the increased action of insulin on the glucose transport system in unweighted rat skeletal muscle.

METHODS

Hindlimb suspension. Male Wistar rats (Sasco, Omaha, NE) weighing 90–100 g were tranquilized with an intramuscular injection (10 μl/100 g body wt) of Innovar-Vet (Pitman-Moore, Mundelein, IL). The experimental groups were tail-casted with Hexalite orthopedic tape and Dow Corning Silastic 382 medical grade elastomer (Factor II; Lakeside, AZ) and suspended as described previously (17) for 3 or 6 days. Food was restricted to 4 g/rat 15 h before the experiment. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt) and treated as described below.

Measurement of in vitro glucose transport activity. The soleus muscles were removed, weighed, prepared in strips weighing ~18 mg (9, 13), and kept at resting length (~15 mm) with stainless steel holders. Muscles were incubated at 37°C for 60 min in stoppered Erlenmeyer flasks containing 2 ml of oxygenated Krebs-Henseleit bicarbonate buffer (KHB) (18) supplemented with 8 mM glucose, 32 mM mannitol, and 0.1% bovine serum albumin (radioimmunoassay grade) in the absence or presence of either 13.3 nM porcine insulin (Eli Lilly, Indianapolis, IN), 20 nM IGF-I (generously supplied by Lilly Research Laboratories, Indianapolis, IN), 5 mM Na_3VO_4 (Fisher Scientific, Santa Clara, CA), or 0.5 U/ml of PLC from *Clos-*

TABLE 1. *Final body weights and soleus wet weights of control and 3- and 6-day suspended animals*

Group	Body Weight, g	Soleus Wet Weight, mg/100 g body wt	Difference From Control, %
Control	96±2	49.0±0.6	
3-Day suspended	97±5	42.0±0.9*	−14
6-Day suspended	102±2	34.0±0.6*†	−31

Values are means ± SE; n = 14–30 rats/group. * $P < 0.001$, 3- or 6-day suspended vs. control; † $P < 0.001$, 6-day vs. 3-day suspended.

tridium perfringens (type XIV, Sigma Chemical, St. Louis, MO).

After the initial treatments, all muscles were rinsed for 10 min at 37°C in oxygenated KHB containing 40 mM mannitol and any previous additions. The muscles were then transferred to flasks containing oxygenated KHB, 1 mM 2-[1,2-^{3}H]DG (300 μCi/mmol), and 39 mM [U-^{14}C]-mannitol (0.8 μCi/mmol; ICN Radiochemicals, Irvine, CA) and any previous additions. After this final 20-min incubation, muscles were trimmed of connective tissue, frozen between aluminum blocks cooled to the temperature of liquid N_2, weighed, and dissolved in 0.5 ml of 0.5 N NaOH. After solubilization, 5 ml of scintillant were added and samples were analyzed for radioactivity in the ^{3}H and ^{14}C channels. The radioactivity in the ^{14}C channel and the specific activity of the incubation medium were used to determine the extracellular space, whereas the specific uptake of 2-DG was calculated by subtracting the ^{3}H activity in the extracellular space from the total ^{3}H activity in each sample. All values for in vitro 2-DG uptake are expressed as picomoles of 2-DG per milligram muscle wet weight per 20 min.

Statistics. The significance of differences between groups was assessed by factorial analysis of variance with a post hoc Scheffé's F test (Statview, Brainpower, Calabasas, CA).

RESULTS

Body weights and soleus muscle wet weights. Final body weights and soleus muscle wet weights of the control, 3-day, and 6-day suspended rats are presented in Table 1. Final body weights were not statistically different. After 3 and 6 days of unweighting by hindlimb suspension, soleus wet weights were 14 and 31% less ($P < 0.001$), respectively, than weight-bearing control muscles. This agrees well with previous studies using similar periods of soleus unweighting (26).

Effect of unweighting on in vitro glucose transport activity. With the use of soleus strips of approximately equal size (see legends to Figs. 1–4) isolated from weight-bearing control rats and rats suspended for 3 or 6 days, 2-DG uptake was determined in vitro in the absence or presence of insulin and a variety of insulin-like factors. No significant differences among groups were found for basal 2-DG uptake (Figs. 1–4). The maximal response for soleus 2-DG uptake to either insulin (Fig. 1) or IGF-I (Fig. 2) was substantially and progressively enhanced by unweighting relative to control muscle. After 3 days of unweighting, insulin-stimulated 2-DG uptake was 34% greater and IGF-I-stimulated 2-DG uptake was 67% greater than in control muscle. After 6 days, these rates of 2-DG uptake were 75 and 156% greater, respectively, than in control muscle.

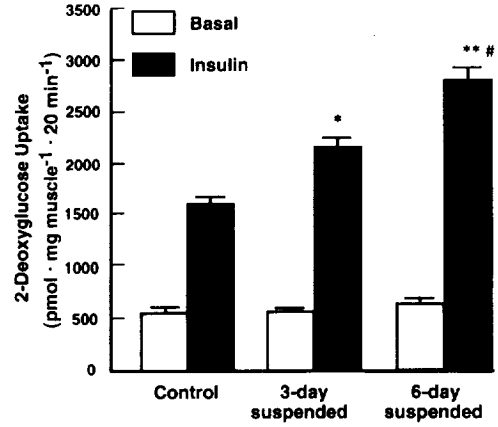

FIG. 1. Effect of unweighting on stimulation of glucose transport activity in soleus muscles by insulin. 2-Deoxyglucose (2-DG) uptake was determined in soleus strips in absence (open bars) or presence (solid bars) of 13.3 nM insulin as described in METHODS. Muscle strip sizes were 17.8 ± 1.2 mg for control group, 17.6 ± 1.0 mg for 3-day suspended group, and 17.1 ± 0.8 mg for 6-day suspended group. Values are means ± SE for 5–6 muscles/group. * $P < 0.05$, ** $P < 0.001$ vs. control + insulin; # $P < 0.01$ vs. 3-day suspended group + insulin.

To investigate potential changes in insulin action via postreceptor events, PLC and vanadate were used to activate glucose transport activity. The maximal response to PLC for stimulation of 2-DG uptake after unweighting is shown in Fig. 3. After 3 days of unweighting, the rate of 2-DG uptake after stimulation by this compound was 36% greater than in control muscle, whereas after 6 days of unweighting PLC-stimulated 2-DG uptake was 74% greater than in control muscle and 28% greater than in the 3-day unweighted muscle.

In contrast to PLC, the maximal response to vanadate for stimulation of 2-DG uptake was unchanged in the 3-day unweighted soleus relative to control muscle (Fig. 4). However, after 6 days of unweighting, the maximal response to this agent was 78% greater than in control

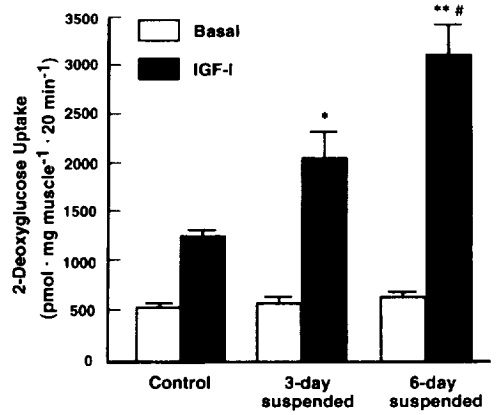

FIG. 2. Effect of unweighting on stimulation of glucose transport activity in soleus muscles by insulin-like growth factor I (IGF-I). 2-DG uptake was determined in soleus strips in absence (open bars) or presence (solid bars) of 20 nM IGF-I. Muscle strip sizes were 17.8 ± 0.6 mg for control group, 19.9 ± 0.7 mg for 3-day suspended group, and 18.1 ± 0.6 mg for 6-day suspended group. Values are means ± SE for 5–10 muscles/group. * $P < 0.05$, ** $P < 0.001$ vs. control + IGF-I; # $P < 0.01$ vs. 3-day suspended group + IGF-I.

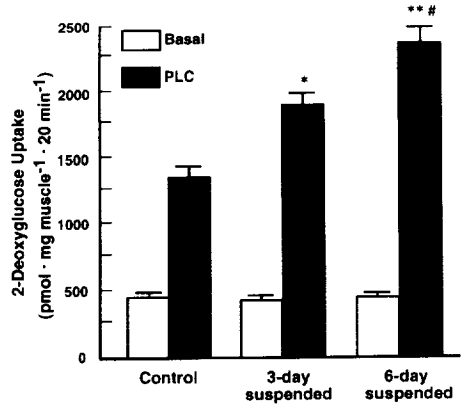

FIG. 3. Effect of unweighting on stimulation of glucose transport activity in soleus muscles by phospholipase C (PLC). 2-DG uptake was determined in soleus strips in absence (open bars) or presence of 0.5 U/ml of PLC (solid bars) as described in METHODS. Muscle strip sizes were 17.8 ± 0.9 mg for control group, 17.8 ± 0.5 mg for 3-day suspended group, and 17.5 ± 1.1 mg for 6-day suspended group. Values are means ± SE for 6–14 muscles/group. * $P < 0.05$, ** $P < 0.001$ vs. control + PLC; # $P < 0.05$ vs. 3-day suspended group + PLC.

muscle and 60% greater than the rate of 2-DG uptake in the 3-day unweighted soleus.

The percent differences from control for 2-DG uptake in unweighted soleus muscle stimulated by insulin and the various insulin-like factors are summarized in Table 2.

DISCUSSION

In the present study, we present evidence that insulin receptor-independent mechanisms play a major role in the enhanced response to insulin that develops in unweighted soleus muscle. This evidence is provided by the following findings. First, unweighting of the soleus resulted in a progressively increased response of glucose transport activity to IGF-I (Fig. 1). IGF-I is thought to act via its own receptor system in skeletal muscle, as

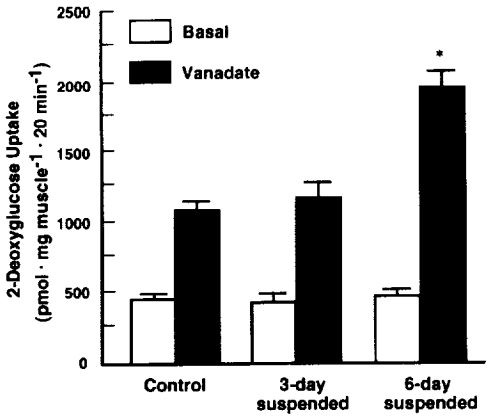

FIG. 4. Effect of unweighting on stimulation of glucose transport activity in soleus muscles by vanadate. 2-DG uptake was determined in soleus strips in absence (open bars) or presence of 5 mM vanadate (solid bars) as described in METHODS. Muscle strip sizes were 18.2 ± 1.3 mg for control group, 16.9 ± 0.6 mg for 3-day suspended group, and 18.5 ± 0.8 mg for 6-day suspended group. Values are means ± SE for 10–20 muscles/group. * $P < 0.001$ vs. control + vanadate.

TABLE 2. *Percent difference for 2-DG uptake in unweighted soleus muscle stimulated by insulin and insulin-like factors*

	Difference From Control, %		Unweighting Effect P Value	
Factor	3-Day	6-Day	3-Day	6-Day
Insulin	+34	+67	<0.05	<0.001
IGF-I	+75	+156	<0.05	<0.001
PLC	+36	+74	<0.05	<0.001
Vanadate	+11	+78	NS	<0.001

2-DG, 2-deoxyglucose; IGF-I, insulin-like growth factor I; PLC, phospholipase C. NS, not significant.

IGF-I has a very low affinity for the insulin receptor and insulin has a very low affinity for the IGF-I receptor in this tissue (6). The maximal effects of insulin and IGF-I on the glucose transport system in muscle are not additive (19, 21), indicating that the actions of these two hormones are mediated by a common intracellular mechanism. It is therefore possible that unweighting alters this intracellular signaling mechanism such that the actions of both insulin and IGF-I are enhanced.

Second, the in vitro action of PLC on the glucose transport system was progressively increased by unweighting (Fig. 2). PLC activates glucose transport in skeletal muscle independently of insulin receptor tyrosine kinase activation by stimulating the formation of diacylglycerol from membrane phospholipids (22), with the subsequent activation of protein kinase C (12). Riley et al. (20) have recently demonstrated that unweighting of the rat soleus results in an abnormal folding of the sarcolemmal membrane, resulting in a redundant membrane architecture. One possibility, which remains to be experimentally tested, is that this membrane restructuring also increases the relative concentration of membrane phospholipids. If this were the case, it would be likely that the enhanced capacity for diacylglycerol generation could contribute to the greater stimulatory capacity of PLC, and perhaps of insulin, in the unweighted soleus.

Third, the stimulatory effect of vanadate on the glucose transport system was enhanced in 6-day unweighted soleus muscle (Fig. 3). Vanadate activates glucose transport in skeletal muscle by using an insulin-dependent mechanism (8, 14) but at a site distal to the insulin receptor (7), possibly involving inhibition of membrane phosphotyrosyl-protein phosphatases (25). Therefore, the enhanced action of vanadate in the 6-day unweighted soleus may involve this postreceptor mechanism. As the vanadate effect was not increased relative to the control muscle in the 3-day unweighted muscle, this suggests that this particular postreceptor alteration developed between *days 3* and *6* of unweighting. Moreover, this finding also provides indirect evidence that multiple adaptations in the unweighted muscle ultimately contribute to the total increase in insulin (and IGF-I) action on the glucose transport system.

In weight-bearing control soleus muscle, the in vitro rate of maximal IGF-I-stimulated 2-DG uptake was only 75% of the rate measured in muscle stimulated maximally with insulin (Fig. 1), whereas in situ the IGF-I-stim-

ulated rate of 2-DG uptake was ~80% of that seen with insulin stimulation (Fig. 4). This comparison is similar to that seen previously for in vitro 2-DG uptake in the epitrochlearis (10). However, these differences in insulin- and IGF-I-stimulated rates of 2-DG uptake disappeared in the 3- and 6-day unweighted soleus (Fig. 1). This suggests that unweighting causes an interaction of the insulin and IGF-I signaling pathways that is normally not present in weight-bearing muscle. A similar response has been reported for hormone-stimulated 2-DG uptake in the epitrochlearis muscle after an exhaustive bout of swim exercise (10).

Differences in food consumption and therefore differences in the metabolic status of the animals can be confounding factors in the interpretation of the responses of skeletal muscle to various interventions. Several lines of evidence indicate that this is not a concern in the present study. First, previous studies using this tail-cast suspension model have shown that food consumption does not differ between suspended and control animals during the first 6 days of treatment (17). Although we did not measure food consumption in the present study, all rats received the same amount of food (4 g) over the last 15 h before the experiment (see METHODS). Second, the atrophic response of the soleus is a local phenomenon resulting from mechanical unweighting, as the extensor digitorum longus, an anterior leg muscle with an activity pattern that is not markedly altered by this intervention, grows normally during suspension (26) and does not display an enhanced response to insulin for glucose transport (15, 16). In addition, it should be emphasized that the muscle weights reported in this paper are normalized to body weight (Table 1), thereby taking into account any differences in body weights between groups.

We do not believe that muscle hypoxia was an issue in these studies. It is well established that isolated solei, such as the ones used in the present study, remain metabolically viable over the incubation period used, as high energy phosphates are maintained (4, 5). In addition, the physiological viability is demonstrated by the substantial increase in insulin-stimulated glucose transport reported here and by the ability of the muscle to contract in response to electrical stimulation, as reported previously (9, 11). Finally, substantial increases in glucose transport activity above basal are achieved in these strips by a truly hypoxic stimulus (11). The fact that the muscle strip sizes in all of the groups studied were the same, as indicated above, actually makes the point moot, as muscle incubation conditions were therefore the same for all muscles.

In summary, the present report provides evidence that soleus unweighting by hindlimb suspension induces an enhancement not only in the action of insulin but also in the action of IGF-I on the glucose transport system, as assessed by in vitro methodology. Furthermore, the enhanced effects of insulin mimickers in unweighted muscle suggest that multiple sites of adaptation that include postreceptor mechanisms must be involved in the increased effect of these hormones on glucose transport activity.

We thank Brett P. Leonard for excellent technical assistance and Dr. Marc E. Tischler for a critical reading of the manuscript.

This work was supported in part by National Aeronautics and Space Administration Grant NAG2-782, by Biomedical Research and Support Grant S07-RR-07002 from the National Institutes of Health, and by the University of Arizona Heart Run Fund.

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Received 2 October 1992; accepted in final form 30 March 1993.

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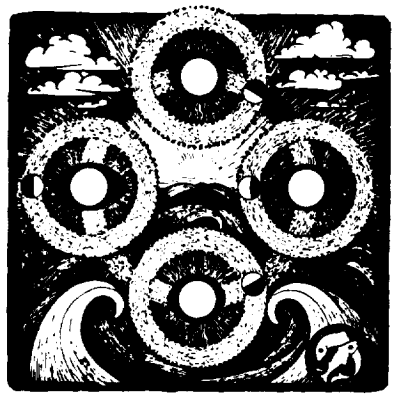

Effect of soleus unweighting on stimulation of insulin-independent glucose transport activity

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HENRIKSEN, ERIK J., AND LESLIE S. RITTER. *Effect of soleus unweighting on stimulation of insulin-independent glucose transport activity.* J. Appl. Physiol. 74(4): 1653–1657, 1993.—Unweighting of the rat soleus by tail-cast suspension results in increased insulin action on stimulation of glucose transport, which can be explained, at least in part, by increased insulin binding and enhanced glucose transporter protein levels. Glucose transport is also activated by an insulin-independent mechanism stimulated by in vitro muscle contractions or hypoxia. Therefore, the purpose of this study was to determine if soleus unweighting leads to an enhanced response of the insulin-independent pathway for stimulation of glucose transport. The hindlimbs of juvenile male Wistar rats were suspended by a tail-cast system for 3 or 6 days. Glucose transport activity in isolated soleus strips (~18 mg) was then assessed by using 2-deoxy-[1,2-^{3}H]glucose (2-DG) uptake. Insulin (2 mU/ml) had a progressively enhanced effect on 2-DG uptake after 3 and 6 days of unweighting (+44 and +72% vs. control, respectively; both $P < 0.001$). At these same times, there was no difference between groups for activation of 2-DG uptake by maximally effective treatments with contractions (10 tetanuses), hypoxia (60 min), or caffeine (5 mM). These results indicate that the enhanced capacity for stimulation of glucose transport after soleus unweighting is restricted to the insulin pathway, with no apparent enhancement of the insulin-independent pathway.

skeletal muscle; 2-deoxy-[1,2-^{3}H]glucose uptake; insulin; hypoxia; caffeine; simulated weightlessness

GLUCOSE TRANSPORT into skeletal muscle is acutely stimulated by an insulin-dependent mechanism involving the translocation of a muscle/fat-specific glucose transporter isoform (GLUT-4) from an intracellular pool to the plasma membrane (6, 15, 20, 30). Glucose transport is also activated by an insulin-independent mechanism that is activated in vitro by electrically stimulated muscle contractions (3, 5, 9, 16, 23, 29), by substances inducing contractions such as caffeine (3, 17, 31), or by exposure to a hypoxic medium (3, 4). Numerous studies have demonstrated that the maximal effects of both pathways on the stimulation of in vitro glucose transport are completely additive, indicating that the stimulatory effects of insulin and contractions are mediated by different mechanisms (5, 9, 23, 29).

Unweighting of the rat soleus muscle by hindlimb suspension has been used as a terrestrial model of weightlessness and induces numerous functional and metabolic adaptations (as reviewed recently in Ref. 28). Unweighting results in rapid muscle atrophy (28), and the onset of significant muscle atrophy is accompanied by an increase in insulin-stimulated glucose transport (2, 11–13, 27). This enhanced insulin-dependent glucose transport activity is likely caused by the development of increased insulin binding (2, 13) as well as by a roughly proportional increase in the expression of GLUT-4 protein (10).

To date, no study has addressed the effect of soleus unweighting on either contraction-dependent stimulation of glucose transport or the combined effects of insulin and contractile activity to stimulate this process. In view of the enhanced expression of GLUT-4 protein that develops in unweighted soleus muscle, we hypothesized that this intervention would lead to increases in both the insulin-independent pathway for activation of glucose transport as well as the ability of insulin and contractions together to stimulate this process. Therefore, the purpose of the present study was to test this hypothesis in soleus muscles unweighted for 3 or 6 days by means of a tail-cast suspension model.

METHODS

Hindlimb suspension. Male Wistar rats (Sasco, Omaha, NE) weighing 90–100 g were tranquilized with an intramuscular injection (10 μl/100 g body wt) of Innovar-Vet (Pitman-Moore, Mundelein, IL). The experimental groups underwent tail-casting with the use of Hexalite orthopedic tape and a Dow Corning Silastic 382 medical grade elastomer (Factor II, Lakeside, AZ) and were suspended as described by Jaspers and Tischler (18) for 3 or 6 days. Food was restricted to 4 g/animal 15 h before the experiment. Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). The soleus muscles were removed, weighed, prepared in strips weighing ~18 mg (9, 10), and kept at resting length with stainless steel holders.

Insulin treatment. Muscles were incubated at 37°C for 20 min in stoppered Erlenmeyer flasks containing 2 ml of oxygenated Krebs-Henseleit bicarbonate buffer (KHB; Ref. 22) supplemented with 8 mM glucose, 32 mM mannitol, and 0.1% bovine serum albumin (radioimmunoassay grade) in the absence or presence of 2 mU/ml porcine insulin (Eli Lilly, Indianapolis, IN).

Muscle contractions, hypoxia, and caffeine treatments. For electrical stimulation of muscle contractions, the distal tendon of the muscle was attached to a vertical Lucite rod containing two platinum electrodes (16). The proximal end was clipped to a jeweler's chain and attached to a Grass model FTO3 isometric force transducer. The mounted muscle was immersed in 25 ml KHB

TABLE 1. *Effect of 3 or 6 days of hindlimb suspension on soleus muscle wet weights*

Group	Soleus Wet Weight, mg/100 g body wt	Difference From Control, %
Control	50.3±1.0	
3-Day suspended	42.3±1.2*	−16
6-Day suspended	33.0±1.1*†	−34

Values are means ± SE for 11–20 muscles/group. * $P < 0.001$ vs. control; † $P < 0.001$, 3-day suspended vs. 6-day suspended.

containing 8 mM glucose and 32 mM mannitol and continuously oxygenated with 95% O_2-5% CO_2 at 37°C. The muscle was stimulated with supramaximal square-wave pulses of 0.2-ms duration by using a Grass S11 stimulator. Ten tetanic contractions were produced by stimulating the muscle at 50 Hz for 10 s at a rate of 1 contraction/min for 10 min. This protocol maximally activates the insulin-independent pathway for glucose transport in the soleus muscle (9).

For maximal hypoxia-stimulated glucose transport activity, muscles were incubated at 37°C for 60 min in KHB containing 8 mM glucose and 32 mM mannitol and gassed with 95%N_2-5%CO_2 (3). Maximal caffeine stimulation of glucose transport activity was accomplished by incubating muscles at 37°C for 60 min in KHB containing 8 mM glucose, 32 mM mannitol, and 5 mM caffeine (3, 17, 31).

Measurement of glucose transport activity. After the initial treatments, all muscles were rinsed for 10 min at 37°C in oxygenated KHB containing 40 mM mannitol and, for insulin-stimulated muscles only, 0.1% bovine serum albumin and 2 mU/ml insulin. The muscles were then transferred to flasks containing oxygenated KHB, 1 mM 2-deoxy-[1,2-^{3}H]glucose (2-DG, 300 μCi/mmol), and 39 mM [U-^{14}C]mannitol (0.8 μCi/mmol; ICN Radiochemicals, Irvine, CA). After this final 20-min incubation, muscles were trimmed of connective tissue, frozen between aluminum blocks cooled to the temperature of liquid N_2, weighed, and dissolved in 0.5 ml of 0.5 N NaOH. After complete solubilization, 5 ml of scintillant were added, and samples were analyzed for radioactivity in the ^{3}H and ^{14}C channels. The radioactivity in the ^{14}C channel and the specific activity of the incubation medium were used to determine the extracellular space, and the specific uptake of 2-DG was calculated by subtracting the ^{3}H activity in the extracellular space from the total ^{3}H activity in each sample. This method for assessing glucose transport activity in the split soleus has been thoroughly studied and validated (26). All values for 2-DG uptake are expressed as picomoles of 2-DG per milligram muscle wet weight per 20 min.

Statistics. The significance of differences between groups was assessed by analysis of variance with a post hoc Scheffé's F test.

RESULTS

Effect of unweighting on muscle mass. As shown in Table 1, after 3 and 6 days of unweighting by hindlimb suspension, soleus wet weights were 16 and 34% less ($P < 0.001$), respectively, than that of weight-bearing control muscles. This finding agrees well with previous studies using similar periods of soleus unweighting (28).

Effect of unweighting on insulin-dependent glucose transport activity. In the absence of stimulus, there were no significant differences in the rates of 2-DG uptake between control muscles and 3- or 6-day unweighted soleus muscles (Fig. 1). In the presence of a maximally effective concentration of insulin (2 mU/ml), 2-DG uptake was 44% greater ($P < 0.01$) after 3 days of unweighting and 72% greater ($P < 0.001$) after 6 days of unweighting than that of control muscles (Fig. 1). Therefore, using soleus strips of similar wet weights in the present study, we have confirmed our previous findings (11–13) with intact soleus muscles that soleus unweighting induces a rapid increase in insulin action on in vitro glucose transport activity.

Effect of unweighting on insulin-independent glucose transport activity. We initially studied the effect of unweighting on the insulin-independent pathway for glucose transport by stimulating soleus muscles electrically to contract in vitro. In stark contrast to the progressively enhanced effect of insulin on 2-DG uptake in the unweighted soleus (Fig. 1), this maximally effective contraction stimulus had no greater effect on 2-DG uptake in the unweighted muscles than in the control muscles (Fig. 2).

There were no significant differences in maximal tetanic tension during electrical stimulation between control muscles (0.66 ± 0.04 g/mg muscle, $n = 21$) and 3-day (0.74 ± 0.06 g/mg muscle, $n = 16$) or 6-day (0.54 ± 0.03 g/mg muscle, $n = 14$) unweighted soleus muscles. Therefore, this variable could not account for the lack of an enhanced capacity of the contraction pathway for stimulation of glucose transport activity in the suspended groups. The small decline in maximal tetanic tension relative to muscle mass after 6 days of soleus unweighting (−18% vs. control) is consistent with findings from previous studies (−4 to −29%) using a similar suspension period (7, 8, 14). We did observe, however, that maximal tetanic tension in the 6-day unweighted soleus was significantly smaller (−27%, $P < 0.05$) than that in the 3-day unweighted muscles.

Hypoxia stimulates glucose transport by the same

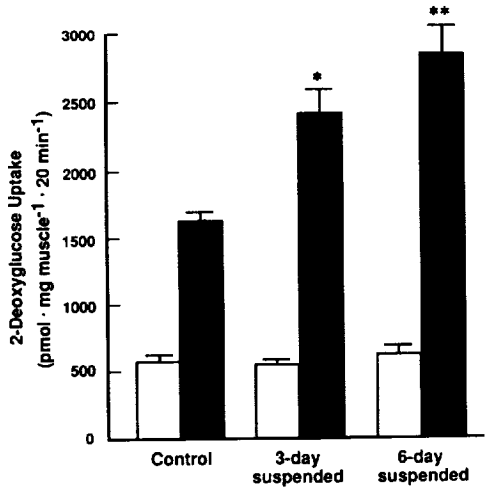

FIG. 1. Effect of 3 or 6 days of suspension on stimulation of glucose transport activity in soleus muscles by insulin. Glucose transport activity, as determined in soleus strips by 2-deoxy-[1,2-^{3}H]glucose uptake, was measured in absence (open bars) or presence (solid bars) of 2 mU/ml insulin as described in METHODS. Values are means ± SE for 9–14 muscles/group. * $P < 0.01$, ** $P < 0.001$ vs. control.

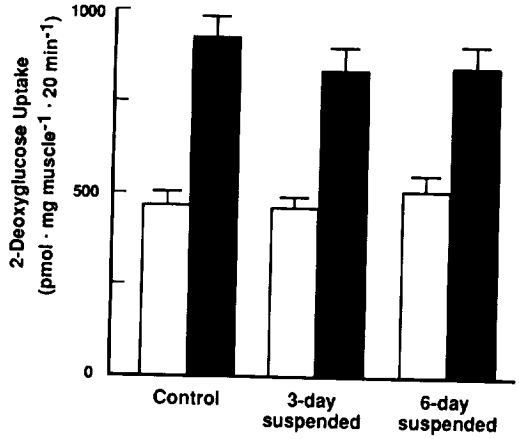

FIG. 2. Effect of 3 or 6 days of suspension on stimulation of glucose transport activity in soleus muscles by contractions. Glucose transport activity was measured in soleus strips receiving either no stimulation (open bars) or 10 electrically induced tetanic contractions (solid bars), as described in METHODS. Values are means ± SE for 9–17 muscles/group.

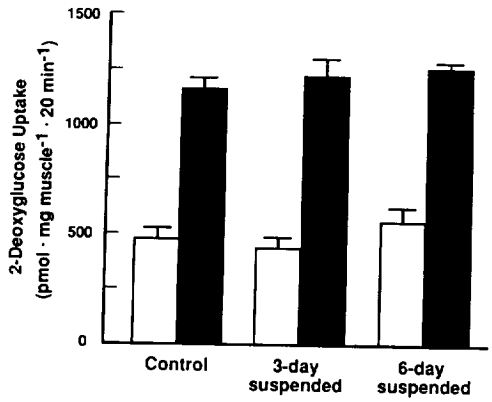

FIG. 3. Effect of 3 or 6 days of suspension on stimulation of glucose transport activity in soleus muscles by hypoxia. Glucose transport activity was measured in soleus strips incubated initially for 60 min in normoxic (open bars) or hypoxic (solid bars) medium, as described in METHODS. Values are means ± SE for 4–9 muscles/group.

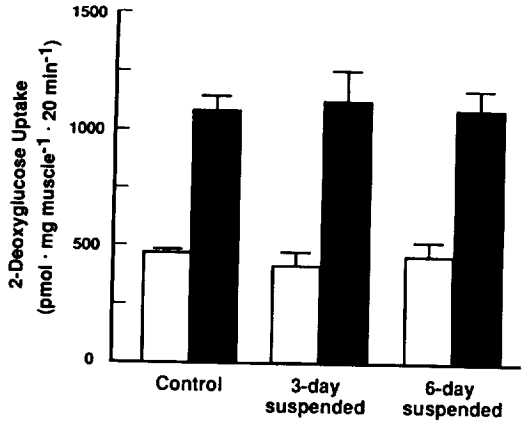

FIG. 4. Effect of 3 or 6 days of suspension on stimulation of glucose transport activity in soleus muscles by caffeine. Glucose transport activity was measured in soleus strips in absence (open bars) or presence (solid bars) of 5 mM caffeine, as described in METHODS. Values are means ± SE for 4–10 muscles/group.

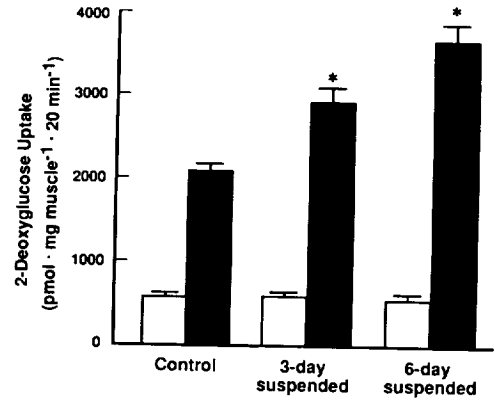

FIG. 5. Effect of 3 or 6 days of suspension on stimulation of glucose transport activity in soleus muscles by insulin and contractions in combination. Glucose transport activity was measured in soleus strips receiving either no stimulation (open bars) or maximal stimulation with insulin (2 mU/ml) plus contractions (10 tetanuses; solid bars), as described in METHODS. Values are means ± SE for 5–8 muscles/group. * $P < 0.01$ vs. control.

pathway as does contractile activity but without causing muscle contraction (3). Therefore, we used hypoxia as a method to further study the insulin-independent pathway for glucose transport in unweighted soleus muscle. As shown in Fig. 3, this maximally effective hypoxic stimulus had no greater effect in the unweighted soleus than in the weight-bearing control muscles.

Caffeine, which activates both muscle contraction (21, 24) and glucose transport activity (3, 17, 31) by releasing Ca^{2+} from the sarcoplasmic reticulum, was used as a pharmacological means by which to study the insulin-independent pathway for glucose transport in the unweighted soleus. This agent also had no greater effect on stimulation of 2-DG uptake in the unweighted muscles than in the control muscles (Fig. 4). There were no statistical differences among groups for the increase in resting tension induced by this concentration of caffeine (control: 96.6 ± 9.0 mg/mg muscle; 3-day unweighted: 91.9 ± 12.5 mg/mg muscle; 6-day unweighted: 77.7 ± 5.7 mg/mg muscle; n = 6–8 muscles/group).

Effect of unweighting on glucose transport activity stimulated by insulin and contractions simultaneously. The maximal effects of insulin and muscle contractions on 2-DG uptake were completely additive in control, 3-day, and 6-day unweighted soleus muscles (Fig. 5). However, this rate of stimulated 2-DG uptake was significantly higher in 3-day (+36%, $P < 0.01$) and 6-day (+74%, $P < 0.001$) unweighted soleus muscles than in control muscles. These differences could be entirely accounted for by the enhanced effect of insulin in the unweighted groups (Fig. 1).

DISCUSSION

The novel finding of the present study was that whereas 3 and 6 days of unweighting by hindlimb suspension resulted in a marked enhancement of insulin action on glucose transport activity in soleus strips of equal size (Fig. 1), at these same times there was no difference relative to the weight-bearing control for activation of the insulin-independent pathway for glucose transport. This latter pathway was stimulated by three independent and maximally effective treatments: electrically stimulated contractions (Fig. 2), hypoxia (Fig. 3), and caffeine (Fig. 4). This indicates that the enhanced capacity for stimulation of glucose transport after soleus unweighting is re-

stricted to the insulin-dependent pathway, with no apparent enhancement of the insulin-independent pathway.

We have demonstrated previously that a strong correlation exists between the GLUT-4 protein level and the in vitro glucose transport activity after maximal stimulation with insulin and contractions in skeletal muscles of widely varying fiber type compositions from young sedentary animals (9). This relationship is maintained in muscles experiencing marked alterations in the chronic level of contractile activity. For example, increased muscle activity induced by either treadmill running (26), voluntary wheel running (25), or swimming (26) results in increased maximal rates of glucose transport activity and is accompanied by significant elevations in the whole muscle level of GLUT-4 protein (25, 26). On the other hand, denervation of the soleus by sciatic nerve sectioning, resulting in the elimination of neural control of muscle activity, leads to decreases in both the maximal rates for glucose transport activity (10) and GLUT-4 protein levels (1, 10). In this latter case, it is noteworthy that alterations in maximal glucose transport activity result from changes in both the insulin-dependent and the insulin-independent pathways (10), whereas after exercise training the maximal glucose transport activity is increased primarily by an enhancement of insulin responsiveness (25, 26).

In a separate study (10), we have demonstrated that whole muscle GLUT-4 protein levels are significantly enhanced after 3 or 7 days of soleus unweighting. It is of note that these increases in GLUT-4 protein levels were roughly proportional to the increases in glucose transport activity stimulated by either insulin alone (Fig. 1) or insulin and contractions in combination (Fig. 5) after comparable periods of unweighting. There are a number of possible interpretations of these results. First, recent evidence suggests that in muscle there are two separate intracellular pools of glucose transporters, one recruited in response to insulin (or insulin-like factors) and the other in response to contractions (6) or hypoxia (3). The present results would be compatible with the hypothesis that unweighting causes an increase in only the insulin-recruitable pool of GLUT-4 protein, with no expansion of the contraction- or hypoxia-recruitable pool.

Alternatively, if a single pool of recruitable glucose transporters exists in skeletal muscle, then under conditions of unweighting the expanded GLUT-4 pool may be accessible only to intracellular signals derived from the insulin-dependent pathway, with some (as yet unidentified) inhibitor preventing accessibility by signals originating from the insulin-independent pathway. Other possible explanations, including the phosphorylation/dephosphorylation state of the GLUT-4 protein or of other intracellular peptides that may potentially be involved in the signal transduction process, cannot be excluded. Elucidation of the actual intracellular mechanisms responsible for our observations will require the development of improved biochemical methodologies for studying both signal transduction and glucose transporter compartmentalization.

In the present study we observed no significant differences among groups for nonstimulated glucose transport activity (Figs. 1–5). This is compatible with our previous observation that unweighting of the soleus does not alter the expression of GLUT-1 protein (10), which has been hypothesized to play a role in the regulation of basal glucose transport in skeletal muscle (19). Interestingly, GLUT-1 protein expression and basal glucose transport activity are also unchanged in muscles that have undergone exercise training (25).

In summary, our results indicate that unweighting of the soleus muscle by tail-cast suspension causes a marked increase in insulin-stimulated glucose transport activity, with no enhancement of the insulin-independent pathway for glucose transport. This model of altered muscle usage, with its discordant adaptive responses for insulin-dependent and insulin-independent glucose transport activity, could be effectively utilized in future research endeavors to examine individually the cellular mechanisms regulating these two pathways for stimulation of glucose transport.

We thank Brett P. Leonard for excellent technical assistance.

This work was supported in part by National Aeronautics and Space Administration Grant NAG2-782 (to E. J. Henriksen), by Division of Research Resources Biomedical Research and Support Grant S07-RR-07002 to (E. J. Henriksen), and by the University of Arizona Heart Run Fund (to L. S. Ritter).

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Received 18 August 1992; accepted in final form 2 November 1992.

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